Remarks

The Applicants acknowledge the earlier election/restriction and reconfirm the earlier election of Group I, including Claims 1 – 4 and 7 – 9. Claims 5 - 7 have been cancelled.

The Applicants note with appreciation the Examiner's helpful comment concerning page 27 of the Specification regarding "Compound 1." The Applicants have amended page 27 at the appropriate location by inserting the correct compound, which is found on page 26. Entry into the Official File is respectfully requested.

The Applicants acknowledge the 35 U.S.C. §112 rejection of Claim 7. Claim 7 has been cancelled, inasmuch as it depends on cancelled Claim 6. The Applicants respectfully submit that the rejection is now moot.

The Applicants have amended Claims 1 – 4 and 7 – 9 to emphasize the neuropathic pain aspect of this invention. The Applicants believe that this is better achieved by amending the claims from "a therapeutic agent for neuropathic pain" to "a method of treating neuropathic pain." This change to the outstanding claims does not affect the scope of the claims or the subject matter intended to be claimed. Entry of these changes into the Official File is respectfully requested.

The Applicants acknowledge the provisional double-patenting rejection over co-pending application 10/477,062. Inasmuch as this is a provisional rejection, the Applicants respectfully request that further treatment of the rejection be held in abeyance pending an indication of allowability of the claims solicited herein or claims solicited in the co-pending application.

The Applicants acknowledge the non-provisional double-patenting rejection over the listed 15 patents. The Applicants respectfully submit that a double-patenting rejection is inapplicable to Claims 1 – 4 and 8 – 9 solicited herein inasmuch as they are directed to a method

of treating neuropathic pain with a pharmaceutical composition comprising as an active ingredient a compound represented by general formula (I) or a pharmaceutically acceptable acid addition salt thereof. The Applicants respectfully submit that all of the indicated 15 U.S. patents do not teach or suggest utilization of the claimed compound in a therapeutically effective amount for neuropathic pain. Withdrawal of the non-provisional double-patenting rejection is respectfully requested.

The Applicants acknowledge the rejection of Claims 1 – 4 and 7 – 9 under 35 U.S.C. §102 as being anticipated by EP '847. (The rejection as it applies to Claim 7 is now moot in view of its cancellation.)

The Applicants have amended independent Claim 1 to recite a method of treating neuropathic pain with the compound represented by general formula (I) or a pharmacologically acceptable acid addition salt thereof which is present in a pharmaceutical composition in a therapeutically effective amount for neuropathic pain. EP '847 does not disclose, teach or suggest a therapeutically effective amount of the compound represented by general formula (I) in a therapeutically effective amount for neuropathic pain. EP '847 provides a wide variety of compounds useful as an analgesic, diuretic, hypertensive sedative, immunoenhancer and anti-HIV agent. However, there is no disclosure of a method of treating neuropathic pain. The Applicants note that neuropathic pain is not applicable to analgesics or vice versa. This is demonstrated in the two attached publications as follows:

> Page 1610, right column, lines 2 – 12 of J.M. Besson, "The neurobiology of pain", THE LANCET, Vol. 353, May 8, 1999, pp. 1610 – 1615; and
>
> Page 24, Fig. 2.1 of Alan Cowan, "Animal Models of Pain", *Novel Aspects of Pain Management: Opioids and Beyond*, Edited by Jana Sawynck and Alan Cowan, Wiley-Liss, Inc., 1999, pp. 21 – 32.

The Applicants therefore respectfully submit that EP '847 is inapplicable to the claims solicited herein. Withdrawal of the rejection is respectfully requested.

In light of the foregoing, the Applicants respectfully submit that the entire Application is now in condition for allowance, which is respectfully requested.

Respectfully submitted,

T. Daniel Christenbury
Reg. No. 31,750

TDC:vbm
(215) 656-3381

The neurobiology of pain

J M Besson

Understanding the plasticity of pain and analgesia exhibited in different pain states may improve therapies for the two major types of pain, neuropathic and inflammatory pain, in which nerve and tissue damage leads to alterations at both peripheral and central levels. At the level of the peripheral nerve, drugs that act on particular sodium channels may target only pain-related activity. Agents that act on some of the peripheral mediators of pain may control peripheral nerve activity. A new generation of non-steroidal anti-inflammatory drugs, cyclo-oxygenase 2 inhibitors, that lack gastric actions are becoming available. In the spinal cord, the release of peptides and glutamate causes activation of multiple receptors, particularly, the N-methyl-D-aspartate receptor for glutamate, which, in concert with other spinal systems, generates spinal hypersensitivity. Blocking the generation of excitability is one approach, but increasing inhibitions may also provide analgesia. Opioid actions are via presynaptic and post-synaptic inhibitory effects on central and peripheral C fibre terminals, spinal neurones, and supraspinal mechanisms. Our knowledge of brain mechanisms of pain is still, however, limited. Other new targets have been revealed by molecular biology and animal models of clinical pain, but the possibility of a "magic bullet" is doubtful. Thus, another approach could be single molecules with dual drug actions, that encompass targets where additive or synergistic effects of different mechanisms may enable pain relief without major adverse effects.

The gate control theory of pain proposed by Melzack and Wall[1] lead to much research in the field of pain. Clinicians welcomed this theory because, from a functional point of view, it explained, or attempted to explain, certain clinical findings that could not be accommodated by previous theories of pain which were far too simplistic.

Since the publication of this theory in 1965, our knowledge of the neurobiology of pain continues to grow, while discoveries in electrophysiology and molecular biology offer glimpses of therapeutic breakthroughs. However, I believe that the gaps between the clinical and basic sciences are becoming wider. To put it simply, basic research is fascinating and flourishes in the public eye, yet too often takes a naive approach to the difficult issues that clinicians are confronted with in terms of providing therapy for certain types of pain. With few exceptions, clinicians have only "old molecules" available with which to treat pain. The partial explanation is that research is difficult and takes a long time, for example, the opioid receptors were formally identified in 1973, but we are still waiting for the development of an opioid with the efficacy of morphine without its side-effects. The best research groups in molecular biology lead the race to clone the three main receptors; μ, δ, and κ.[2] Nevertheless, many questions are unresolved: the classic pharmacological techniques that have been applied to this field suggest that subtypes of the receptors exist, whereas molecular biology has yet to come up with any evidence to support this premise.

Substantial difficulties arise in basic research, and before I sketch out an overview of the neurobiology of pain, I will consider some of these difficulties. Scientists engaged in research need to take a more realistic approach to their results so that clinicians are not lead to believe that many useful treatments for pain are just around the corner.

Lancet 1999; 353: 1610–15

INSERM (U 161), Physiopharmacologie du Système Nerveux and EPHE 2 rue d'Alésia, 75014 Paris, France (J M Besson MD)

Laboratory models

The relevance or not of the major behavioural tests for clinical pain states has been widely debated. Tests used to assess antinociceptive activity in the laboratory include noxious heat, pressure to the tail or paw, colorectal distension, intraperitoneal chemical irritants, and subcutaneous administration of formalin. In most cases, these stimuli are applied to healthy animals in the absence of disorders that commonly occur in patients who experience pain such as hyperalgesia (extreme sensitiveness to painful stimuli), allodynia (pain in response to a non-noxious mechanical stimulus), and hyperesthesia (abnormal sensitivity to sensory stimulus). Some of these tests depend on spinal mechanisms, whereas others involve supraspinal structures. Some tests have good sensitivity for a particular class of analgesics, but other tests frequently produce false-positive results. In addition, many behavioural experiments use only one nociceptive test, and the exact method can vary from investigator to investigator. This situation means that controversy surrounds the pharmacology of pain.

Various genetic approaches have been used in pain research, but the most popular is a laboratory model: the production of transgenic mice. For example, substance P has actions in the periphery and centrally, and mice without substance P (after knockout of the preprotachykinin gene),[3,4] or the neurokinin-1 receptor[5] have been produced. These models are difficult to compare since the first model includes mice without the ligand, substance P, and in the second model the receptor has been knocked out. Nevertheless, in both cases, neurogenic inflammation is substantially diminished, although somewhat surprisingly there is no change in the mechanical hypersensitivity induced by the inflammation.

Woolf and colleagues[6] compared in detail various models of transgenic mice and identified three general factors that are important in terms of the interpretation of these techniques: the genetic background of the animal; the developmental changes that could be encountered; and the redundancy of certain functions of sensory systems. Although there is no doubt that the deletion of receptors, channels, and transmitters by genetic

manipulations produces a powerful tool for the dissection of their roles in complex neuronal systems, the results of genetic approaches need to be interpreted with caution and large-scale studies are needed to complete pharmacological research.

These laboratory and behavioural models are limited because they do not mimic chronic pain states. Chronic pain differs substantially from acute pain in terms of the persistence of the pain and adaptive changes such as neuroplasticity that has been described at various levels of the nervous system. Such limitations have led to the use and development of more appropriate models of chronic pain in the past 10 years. These models include inflammatory pain and neuropathic pain. Although they are not perfect, the development of such experimental models is essential, not only for the detection of new analgesics, but also for a better understanding of pain syndromes that are difficult to manage clinically. Behavioural tests are limited and can be remarkably difficult to carry out properly. Clinicians need to realise, for example, how hard it can be for a researcher, to quantify allodynia by approaching an awake freely moving rat or mouse with a calibrated von Frey hair.

Other difficulties encountered in the development of safe analgesics arise from the complexity of the central nervous system. Some of the transmitters and receptors that may be involved in the transmission or modulation of pain are widely distributed throughout the nervous system, especially in the case of peptides and excitatory or inhibitory aminoacids. Most of these neuroactive substances are involved in multiple physiological functions, and so agents developed to target these systems could produce widespread side-effects. Additional difficulties result from the multiplicity of receptors and the co-localisation of more than one neurotransmitter in a single neuron. A further complexity of the network is that some peptides or excitatory aminoacids, for example substance P and glutamate, are localised not only in primary afferent neurons but also in intrinsic spinal neurons and descending fibers. Thus, caution is needed in interpreting the data and to avoid the temptation of becoming infatuated with the molecule of the moment.

In this paper, I review current knowledge of the different stages in the transmission of noxious messages from the periphery to the brain. Later in this series, Fernando Cervero and Jennifer Laird[7] will examine visceral pain and Clifford Woolf and Richard Martin[8] will explore neuropathic pain.

The peripheral jungle

A widely held assumption is that there is no specific histological structure that acts as a nociceptive receptor and that noxious messages arise from the activation of free unmyelinated terminal arborisations found in cutaneous, muscular, joint tissues, and in certain visceral structures. The nociceptive messages are then transmitted by thin myelinated (Aδ) or non-myelinated (C) fibres, although not all of the fibres are necessarily nociceptors. Studies in animals and human beings have identified various types of nociceptors.[9] Various classification systems have been proposed, for example, in cutaneous tissue in man the existence of unmyelinated polymodal nociceptors, which are responsive to thermal, mechanical, and chemical stimuli (with a slow conduction velocity of <2 m/s) have been established. Similarly, Meyer and colleagues[10] identified Aδ mechanothermal nociceptors and high threshold Aδ mechanoreceptors. Aδ and C nociceptors have been clearly identified in fibres, innervating joints and muscles, but not in viscerae where the situation is much more complicated. Thus, although certain fibres are undoubtedly nociceptors, others are activated by non-noxious stimuli but then increase their activity as the intensity of the stimulus increases.

Receptors localised on primary afferent fibres and their ligands from neuronal and non-neuronal origins

Recptors associated with nociceptors
ATP, neurokinin-1, $GABA_A$, $CABA_B$, neuropeptide Y, acetylcholine, somatostatin, prostaglandin E, cholecystokinin, adrenergic, 5 hydroxytryptamine ($5HT)_{2A}$ receptor, glutamine, bradykinin, noradrenaline, capsaicin, opioid, angiotensin II, adenosine

Ligands with non-neuronal sources
Acetylcholine, ATP, prostaglandin E, opioids, adenosine, glutamate, bradykinin, noradrenaline, serotonin

Ligands in nociceptors
Substance P, opioid, ATP, adenosine, neuropeptide Y, glutamate, cholecystokinin, somatostatin, bombesin

GABA=γ-aminobutyric acid.
Adapted with permission from Carlton and Coggeshall.[13]

Sensitivity

When a stimulus is repeated nociceptors exhibit sensitisation in that there can be a reduction in the threshold for activation, an increase in the response to a given stimulus, or the appearance of spontaneous activity. This sensitisation of nociceptors results from the actions of second messenger systems activated by the release of several inflammatory mediators (bradykinin, prostaglandins, serotonin, histamine).[11] These effects, which seem to be specific to the different groups of nociceptors, cause some of the features of the hyperalgesia produced by pathological processes. Indeed, primary hyperalgesia, which by definition occurs at the site of tissue damage and can also be produced by mechanical and thermal stimuli, accounts for much of the peripheral sensitisation of nociceptors, although some sensitisation seems be due to central mechanisms of hyperexcitability.

Sleeping nociceptors

Another important finding is that many nociceptors cannot normally be activated and become excitable only under pathological conditions such as inflammation. These are the silent or sleeping nociceptors, first described by Schaible and Grubb[12] in joint tissue. These nociceptors have subsequently been found in visceral and cutaneous tissue. This simple example illustrates how classification can be too rigid. The terminals of nociceptors and their microenvironment have been described as a jungle through which a scientist has difficulty in forging a route to find the secrets contained within.

In 1997 Carlton and Coggeshall,[13] summarised the receptors found on afferent fibres (panel), which they described by anatomical, electrophysiological, and pharmacological approaches. The panel includes ligands of neuronal origins contained and released into the periphery by nociceptive fibres and ligands with non-neuronal origins. This long list is in fact even more complex since many receptors can also be separated into subtypes.

Pharmacology

I provide only a brief review of the pharmacological features of peripheral nociception; several in-depth reviews have been published.[13-17] Various chemicals (bradykinin, histamine, serotonin, prostaglandins, potassium, protons) are released into damaged tissue cells of vascular origins (platelets, neutrophils, lymphocytes, and macrophages) and also by mast cells. When injected by the intradermal route, some of these chemicals induce nociceptive reactions and can modify the activity of nociceptors either by direct activation or by sensitisation to different types of stimuli, such as thermal, mechanical, and chemical. Bradykinin, for example, a powerful algogenic substance released from kininogens in the circulation activates nociceptors in a way that is dependent on protein kinase C and calcium and sensitises nociceptors by means of the activation of postganglionic sympathetic neurones which then produce prostaglandin E2.

Several peptides are contained within primary afferent fibres and their profile can be altered by sustained stimuli or by damage to the nerve.[16,18] Although the roles of several of these peptides are unclear (galanin, somatostatin, cholecystokinin, vasoactive intestinal peptide), others such as substance P and calcitonin gene related peptide can be released into the periphery via the classic axon reflex. The role of substance P in neurogenic inflammation has been clearly shown. The peptide causes a degranulation of mast cells and thus the release of histamine, vasodilatation, and plasma extravasation with the subsequent release of other algogens (bradykinin, serotonin) and the activation of other inflammatory cells (macrophages, monocytes, and lymphocytes). Furthermore, substance P is able to induce production of nitric oxide, another vasodilator from the endothelial layer of blood vessels.

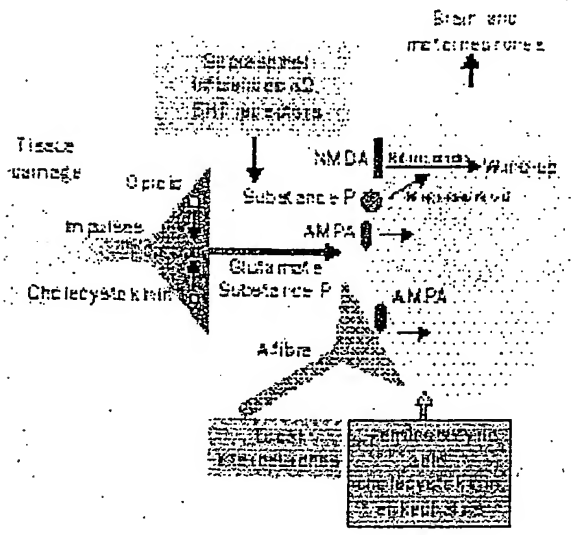
Apart from these substances which, in broad terms, are liberated soon after tissue damage, other factors such as the cytokines (interleukins, interferon, and tumour necrosis factor), are released by phagocyte cells and cells of the immune system and have an important role in the inflammatory process. The role of bradykinin in the sequence of events that lead to the production of the cytokines is well established.[11] Some of these agents are powerful inflammatory mediators that can activate sensory neurones through different mechanisms, some of which include the sympathetic nervous system.[19]

Nerve growth factor has a key role not only in the development of sensory and autonomic neurones, but also in the processes of nociception.[20] This factor, which is upregulated by the process of inflammation, is produced in the periphery by fibroblasts and Schwann cells and then increases the excitability of nociceptors which leads to hyperalgesia. Various central and peripheral mechanisms have been postulated as a basis for these actions of nerve growth factor.[8] The production of antagonists for the receptors—the tyrosine kinase family—has the potential to provide a pharmacological target for the production of new analgesics to reduce the effects of nerve growth factor.

The prostaglandins and probably also the leukotrienes are weak algogens but play a major part in the sensitisation of receptors to other substances. The basis for the analgesic actions of the non-steroidal anti-inflammatory drugs (NSAIDS) is their ability to prevent the production of the prostaglandins. Activation of phospholipase A2 leads to the production of arachidonic acid from membrane phospholipids, which results in the subsequent transformation to thromboxane, the prostacyclins, and the prostaglandins. The main action of NSAIDs is to inhibit the activity of cyclo-oxygenase, the enzyme responsible for the synthesis of the prostaglandins, but this action leads to the production of side-effects. Great hope has been inspired by the characterisation of two isoforms of the enzyme cyclo-oxygenase 1 and 2 (COX-1 and COX-2),[21-23] produced by different genes but with a structural homology of about 60% of the aminoacid residues. However, both the location and regulation of the two isoforms are different. COX-1 is a constitutive enzyme found in endothelial cells, platelets, the mucosa of the stomach, and in the kidney; it is involved in the processes of vascular homeostasis and the regulation of gastric acid and the kidney. Under normal conditions, COX-2 is not found in tissues such as prostatic and lung tissue but can be produced by different signals from hormones, growth factors, mitogens, inflammatory mediators (cytokines), and endotoxins (lipopolysaccharides). Thus, the expression of COX-2 will link prostaglandin synthesis to inflammatory processes. The synthesis of selective inhibitors of COX-2 is an important pharmacological goal in terms of the production of NSAIDs without the side-effects of the present agents. Some laboratories have produced inhibitors of COX-2 the first of which are already available for use in human beings. The anti-inflammatory and antinociceptive effects of these agents seem to be equivalent to those of mixed inhibitors, but the main advantage of the new inhibitors will be the absence of gastric side-effects in patients with chronic pain of inflammatory origins.

Molecular and genetic approaches have lead to a revolution in physiological and pharmacological research in pain, especially at the peripheral level. The cloning of various receptors have advanced our understanding of the mechanisms of transduction and sensitisation. Major breakthroughs include the first cloning of a receptor for capsaicin, the active ingredient of chilli peppers,[24,25] and the receptors for the purines, notably the P2X3 (a ligand-gated ion channel triggered by ATP) which is selectively expressed by small-diameter sensory neurons.[26] Another is the acid-sensing ion channel that is rapidly activated by conditions of acidity below pH 6·5[27] and the tetrodotoxin-resistant sodium channel.[28] Inflammatory mediators, such as prostaglandin E2, adenosine, and serotonin facilitate transmission of action potentials by modification of the voltage threshold of several ion channels, including the tetrodotoxin-resistant sodium channel. Research is in progress for the production of sodium-channel blockers with greater specificity than existing agents so that they would not have the cardiac and central-nervous-system depressant effects that limit the use of present agents.

Thus, it is clear that there are many encouraging approaches that could lead to the production of peripherally acting analgesic drugs that do not pass the blood brain barrier and so lack central side-effects. Another encouraging possibility is that the biological prediction of the structure of macromolecules will allow the three-dimensional structures of receptors to be elucidated, which in turn could lead to the rational development of agonists and antagonists with great specificity and few side-effects. Many substances with neuronal and non-neuronal origins act at the peripheral



Interactions between different excitatory and inhibitory systems in the spinal cord
Adapted with permission from Dickenson[25]

level to modulate the activity of nociceptors and various interactions can occur between these mediators. So would the modulation of only one of these substances sufficient to alter the level of pain in the periphery—could there be a magic bullet with peripheral actions only? This option is unlikely on the basis of current pharmacological information. Only an in-depth analysis of the physiopathology of the different syndromes that originate from peripheral processes can guide a clinician in prescribing the most effective substance. An alternative approach that seems more likely is the production of an analgesic with mixed peripheral actions, so that it acts on different receptor types, or perhaps a move towards a systematic analysis of the effects of administration of several agents.

From spinal cord to brain

The spinal mechanisms of nociception have been studied extensively.[29,30] The detailed characteristics of the neurones of the spinal cord implicated in the transmission of painful messages have been described as the segmental and supraspinal mechanisms that can modulate the information transferred to the brain. But yet again, it is the pharmacological characteristics that attract the attention of research groups. Unfortunately, as with the periphery, the dorsal horn of the spinal cord contains many transmitters and receptors both identified and putative including: several peptides (substance P, calcitonin gene related peptide, somatostatin, neuropeptide Y, and galanin); excitatory aminoacids (aspartate, glutamate); inhibitory aminoacids (γ-aminobytyric acid [GABA] and glycine); nitric oxide; the arachidonic acid metabolites; the endogenous opioids; adenosine; and the monoamines (serotonin and noradrenaline).[11,31,32] This list indicates that there are diverse therapeutic possibilities for the pharmacological control of the transmission of nocicepetive information to the brain. I will address the options related to substance P and glutamate. Since release of substance P is blocked by morphine at the trigeminal level,[33] one would expect this peptide to be one of the principal neurotransmitters released by primary afferent nociceptive fibres at the level of the spinal cord. Although some, although not all, studies show antinociceptive effects of antagonists of the receptor for substance P, the neurokinin-1 receptor in animals, the clinical studies have been disappointing. Furthermore, Mantyh and colleagues' finding[34,35] of stimulus-evoked internalisation of the neurokinin-1 receptor in the spinal cord raises a number of questions. If there is a mismatch at several sites between the localisation of substance P and the receptor, why is it that after the internalisation caused by a noxious stimulus, the receptors do not return to the neuronal membrane until 1 h later? Why does morphine not alter the internalisation, whereas agonists at the GABA B receptor do?

Further questions emerge when one considers the conflicting findings of studies based on different experimental approaches. Thus, Mantyh and colleagues[35] found that selective destruction of the neurones in the superficial spinal cord that express the neurokinin-1 receptor lead to a substantial reduction in allodynia and hyperalgesia induced by inflammation and nerve injury. These findings do not accord with the genetic studies that showed knockout of the preprotachykinin gene or the neurokinin-1 gene lead to only minor changes in the mice. Thus, whether substance P is indeed an important factor in spinal transmission is not known. Perhaps it is not surprising that clinical studies with antagonists of substance P in migraine, pain after dental surgery, in rheumatoid arthritis, and posthepatic neuralgia have been unsuccessful.[36]

The excitatory aminoacids (notably glutamate) are not only the major class of excitatory transmitter in the central nervous system, but are released by primary afferent fibres and have an important role in the spinal mechanisms of pain transmission. Various receptors and subtypes are involved at the spinal level (AMPA, metabotrophic, kainate), but it is the N-methyl-D-asparate (NMDA) receptor that has attracted most attention.

The NMDA receptor is important in the synaptic events that lead to central sensitivity and hyperalgesia.[31,32] The release of peptides such as substance P into the spinal cord on afferent stimulation removes the magnesium block of the channel of the NMDA receptor and thus allow glutamate to activate the NMDA receptor in a range of persistent pain states. This process, unlike other spinal changes, leads to the generation of spinal hypersensitivity and amplification of peripheral inputs. Furthermore, activation of the NMDA receptor leads to an entry of calcium into the neurone which can then produce other mediators from spinal neurons by increasing the activity of enzymes. For example, nitric-oxide synthase generates a gas, nitric oxide, that acts as a freely diffusible transmitter and in a complex way exacerbates the noxious transmission.[37] The entry of calcium can also activate phospholipases and lead to the spinal production of prostanoids, an effect that may be the basis for the central actions of NSAIDs.[38]

The figure shows some of the other possible targets at the spinal level for the control of the transmission of nociceptive messages. These include GABAergic systems, antagonists of cholecystokinin, the inhibitors of the enzymes that degrade the endogenous opioids, and agonists that act at the opioid receptors.[11,39] Morphine exerts a powerful depressive action directly in the spinal cord,[40,41] which is the basis for the clinical applications of

spinal routes of opioid analgesia. Morphine also acts at the brainstem and midbrain levels to alter the activity of descending control systems that are projected from these sites to the spinal cord. Studies on the direct and indirect spinal actions of morphine started 30 years ago and have emphasised this important site in the production of analgesia. However, few studies have examined supraspinal mechanisms in vivo. The importance of the spinal mechanism (direct or indirect) compared with the actions mediated by supraspinal structures is not known. Numerous regions of the brain are rich in opioid peptides and the mRNAs for the opioid receptors.[42] The supraspinal actions of opioids are commonly underestimated and may have a key role in the analgesic effects of systemic morphine. Finally, for the sake of completeness, Stein's[43] finding of a peripheral antinociceptive site of action of opioids in hyperalgesic inflammatory conditions in mice indicates that the local application of opioid agonists or the systemic administration of agonists that do not cross the blood brain barrier could provide analgesia in certain clinical situations. Some clinical studies lend support to this premise, but it is too early to state definitively that this technique has therapeutic value.

The figure also shows the involvement of descending pathways that use serotonin and noradrenaline to control nociception.[44] Many experimental studies have shown that serotonin is important in pain, yet apart from in headache, the production of many analgesics acting on serotonin (5HT) receptors has been confounded by the number of different types and subtypes of the receptors. By contrast, the pharmacology of the systems that use noradrenaline as a transmitter are much simpler and, so, agonists at the $\alpha 2$ adrenoceptors, such as clonidine, have substantial analgesic effects in animals and lesser but still obvious effects in man. However, the agonists at the $\alpha 2$ receptor possess important side-effects and so adrenergic receptor agonists which have improved potency and selectivity are the focus of research based on subtypes of the receptors.

The myriad substances implicated at the spinal level in the transmission and modulation of pain messages leads to the same question that arises at the peripheral level. Is it realistic to expect the development of a single magic bullets or would it be possible to produce one molecule with dual pharmacological actions or use a combination of drugs (multimodal analgesia) to elicit synergistic or additive actions of the combination? Many examples exist of this approach, such as the association of morphine with agonists at the $\alpha 2$ receptor or with antagonists at the cholecystokinin and the NMDA receptor. This type of approach has the dual advantages of improved effects and fewer side-effects through use of lower doses of each agent. This example is only one among many combinations other than with morphine. Although this approach is less spectacular than the magic bullet, it could be more beneficial to the patient and could be used as general principle in this research.

Multipe ascending pathways to the brain

Combinations of electrophysiological and anatomical techniques are increasingly used to identify neurones at the origins of the main ascending pathways and also their termination zones at higher centres of the brain.[30,45] These neurones at the origins of the ascending pathways are located in superficial and deep laminae of the dorsal horn. Although some still hold to the idea of specificity of pain pathways,[46] this concept is highly controversial. There are multiple pain pathways, including the classic routes (spinothalamic tract, the different components of the spinoreticular tract), the spinocervicothalamic tract, the postsynaptic dorsal column fibres, and the visceral nociceptive tracts that run in the posterieur columns. Villanueva and Bernard[47] have described how the several ascending pathways, quite different from each other, project to the mesencephalon and the diencephalon.[45] In addition, the activation of long and short propriospinal circuits cannot be excluded, and it must be underlined that in these studies of ascending pain pathways, mechanisms of chronic pain are frequently explained on the basis of studies on nociceptive in acute pain, without taking into account spinal-cord readjustment (plasticity) after the lesions. Systematic studies of patients with different spinal lesions and disorders that can be undertaken with current imaging techniques will provide new information and a better understanding of the physiopathological features of these ascending pathways.

On the basis of this multiplicity of pain pathways, it is not surprising that positron emission tomography or functional magnetic resonance imaging have revealed activation of various brain regions during acute pain.[48] Although the results are fairly consistent in healthy patients, controversies have arisen from studies in patients with chronic pains. These data tend to support the idea that pain is not a unique consequence of impulses in specific, unidirectional hardwired lines that originate in the periphery and terminate in the central nervous system. We are still in the early stages of the exploration of the human brain, but controlled studies will allow the identification of the regions of the brain that are involved in the different components of pain. At these levels in the brain the pharmacological approaches falter, which is the main reason for the major thrust to target new analgesics at the spinal and peripheral levels.

References

1 Melzack R, Wall PD. Pain mechanisms: a new theory. *Science Wash CD* 1965; 150: 971–79.
2 Kieffer BL. Molecular aspects of opioid receptors. In: Dickenson AH, Besson JM, eds. Handbook of experimental pharmacology: the pharmacology of pain. Berlin: Springer, 1997: 281–303.
3 Cao YQ, Mantyh PW, Carlson EJ, Gillepsie AM, Epstein CJ, Basbaum AL. Primary afferent tachykinins are required to experience moderate to intense pain. *Nature* 1998; 392: 334–35.
4 Zimmer A, Zimmer AM, Baffi J, et al. Hypoaklgesia in mice with a targeted deletion of the tachykinin 1 gene. *Proc Natl Acad Sci USA* 1998; 95: 2630–35.
5 De Felipe C, Herrero JF, O'Brien JA, et al. Altered nociception, analgesia and aggression in mice lacking the receptor for substance P. *Nature* 1998; 392: 334–35.
6 Woolf CJ, Mannion RJ, Neumann S. Null mutations lacking substance: eludicating pain mechanisms by genetic pharmacology. *Neuron* 1998; 20: 1063–66.
7 Cervero F, Laird JMA. Visceral pain. *Lancet* (in press).
8 Woolf CJ, Mannion RJ. Neuropathic pain: aetiology, symptoms, mechanisms, and management. *Lancet* (in press).
9 Belmonte C, Cervo F. Neurobiology of nociceptors. Oxford: Oxford University Press, 1996.
10 Meyer RA, Campbell JN, Raja N. Peripheral neural mechanisms of nociception. In: Wall PD, Melzack R, eds. Edinburgh: Churchill Livingstone, 1994: 13–44.
11 Dray A. Kinins and their receptors in hyperalgesia. *Can J Pharmacol* 1997; 75: 704–12.
12 Schaible H, Grubb BD. Afferent and spinal mechanisms of joint pain. *Pain* 1993; 55: 5–54.
13 Carlton SM, Coggeshall RE. Nociceptive integration: does it have a peripheral component? *Pain Forum* 1998; 7: 71–78.

14 Cesare P, McNaughton P. Peripheral pain mechanisms. *Cur Opin Neurobiol* 1977; 7: 493–95.

15 Dickenson AH, Besson JM, eds. The Pharmacology of pain, handbook of experimental pharmacology, vol. 130. Berlin: Springer, 1997.

16 Levine JD, Fields HL, Basbaum AI. Peptides and the primary afferent nociceptor. *J Neurosci* 1993; 13: 2273–86.

17 Rang HP, Urban L. New molecules in analgesia. *Br J Anaesth* 1995; 75: 145–56.

18 Hökfelt T, Zhang X, Wiesenfeld-Hallin Z. Messenger plasticity in primary sensory neurons following axotomy and its functional implication. *Trends Neurosci* 1994; 17: 22–30.

19 Jänig W, Levine JD, Michaelis M. Interactions of sympathetic and primary afferent neurons following nerve injury and tissue trauma. *Prog Brain Res* 1996; 113: 161–84.

20 McMahon SB, Bennett DLH. Growth factors and pain. In: Dickenson AM, Besson JM, eds. Berlin: Springer, 1997: 135–60.

21 Xie W, Chapman JG, Robertson DL, et al. Expression of a mitogen-responsive gene encoding prostaglandin H synthase is regulated by mRNA splicing. *Proc Natl Acad Sci USA* 1991; 88: 2692-96.

22 Picot D, Loll PJ, Garavito N. The X-ray crystal structure of the membrane protein prostaglandin H2 synthase-1. *Nature* 1994; 367: 243–49.

23 Vane JR, Botting RM. New insight into the mode of action of anti-inflammatory drugs. *Inflamm Res* 1995; 44: 1–10.

24 Caterina MJ, Schumacher MA, Tominaga M, Rosen TA, Levine JD, Julius D. The capsaicin receptors: a heat-activated ion channel in the pain pathway. *Nature* 1997; 389: 816–24.

25 Tominaga M, Caterina MJ. Malmberg AB, et al. The cloned capsaicin receptor integrates multiple pain-producing stimuli. *Neuron* 1998; 21: 531–43.

26 Burnstock G, Wood JN. Purinergic receptors: their role in nociception and primary afferent neurotransmission. *Current Opin Neurobiol* 1996; 6: 526–32.

27 Waldmann R, Champigny G, Bassilana F, Heurteaux C, Lazdunski M. A proton-gated cation channel involved in acid-sensing. *Nature* 1997; 386: 173–77.

28 Akopian AN, Sivilotti L, Wood JN. A tetrodotoxin-resistant voltage-gated sodium channel expressed by sensory neurons. *Nature* 1996; 379: 257–62.

29 Besson JM, Chaouch A. Peripheral and spinal mechanisms of nociception. *Physiol Rev* 1987; 67: 67–184.

30 Willis WD, Westlund KN. Neuroanatomy of the pain system and of the pathways that modulate pain. *J Clin Neurophysiol* 1997; 14: 2–31.

31 Dickenson AH. Spinal cord pharmacology of pain. *Br J Anaesth* 1995; 75: 193–200.

32 Urban L, Thompson SWN, Dray A. Modulation of spinal excitability: co-operation between neurokinin and excitatory amino acid neurotransmitters. *TINS* 1994; 17: 432–38.

33 Jessell TM, Iversen IL. Opiate analgesics inhibit substance P release fron rat trigeminal nucleus. *Nature* 1977; 268: 549–51.

34 Mantyh PW, Demaster E, Malhotra A, et al. Receptor endocytosis and dendrite reshaping in spinal neurons after somatosensory stimulation. *Science* 1997a; 268: 1269–1623.

35 Mantyh PW, Rogers SD, Honore P, et al. Inhibition of hyperalgesia by ablation of lamina I spinal neurons expressing the substance P receptor. *Science* 1997b; 278: 275–79.

36 Dray A, Rang H. The how and why of chronic pain states and the what a new analgesic therapies. *TINS* 1998; 21: 315–17.

37 Meller ST, Gebhart GF. NO and nociceptive processing in the spinal cord. *Pain* 1993; 52: 127–36.

38 Malmberg AB, Yaksh TL. Hyperalgesia mediated by spinal glutamate or substance P receptor blocked by spinal cyclooxygenase inhibition. *Science* 1992; 257: 1276–79.

39 Rpques BP, Noble F, Daugé V, Fournié-Zaluski MC, Beaumont A. Neutral endopeptidase 24.11: structure, inihibition and experimental pharmacology. *Pharmacol Rev* 1993; 45: 118–46.

40 Le Bars D, Besson JM. The spinal site of action of morphine in pain relief: from basic research to clinical applications. *Trends Pharmacol Sci* 1981; 2: 323–25.

41 Yaksh TL. Spinal opiate analgesia: characteristics and principles of action. *Pain* 1981; 11: 293–346.

42 Mansour A, Fox CA, Akil H, Watson SJ. Opiod-receptor mRNA expression in the rat CNS: anatomical and functional implications. *Trends Neurosci* 1996; 18: 22–29.

43 Stein C, Schäfer M, Cabot PJ, Zhang L, Carter L. opiods and inflammation. In: Borsook D, ed. Molecular neurobiology of pain, progress in pain research and management. Seattle: IASP Press, 1997; 9: 25–43.

44 Basbaum AI, Besson JM, eds. Towards a new pharmacology of pain. London: John Wiley & Sons, 1991.

45 Besson JM, Guilbaud G, Ollat H, eds. Forebrain areas involved in pain processing. Paris: John Libbey Eurotext, 1995.

46 Perl E. Gettinf a line on pain: is it mediated by dedicated pathways? *Nature Neurosci* 1998; 1: 177–78.

47 Villanueva L, Bernard JF. The multiplicity of ascending pain pathways. In: Lydic R, Baghdoyan HA, eds. Handbook of behavioural state control, cellular and molecular mechanisms, Boca Raton: CRC Press, 1999; 569–85.

48 Derbyshire SWG, Jones AKP, Gyulai F, Clark S, Townsend D, Firestone LL. Pain processing during three levels of noxious stimulation produced differential patterns of central activity. *Pain* 1997; 73: 431–45.

rsal horn and effects of descending

cid transmitters: Ann Rev Pharmacol

rssmann WG, Reinecke M, Tschopp
tonin gene-related peptide and sub-
al cord and interact in spinal behav-
)–204.
nisms of the Spinal Cord. New York:

al mechanisms. In Wells JCD, Woolf
rchill Livingstone, pp. 523–533.
alteration of C-fibre evoked activity
al cord. Neurosci Lett 25:37–41.
elates of the tactile evoked allodynia
of modulatory receptor systems and

n E, A (ed): *Opioids II. Handbook*
. 53–90.
ffects of a nonpeptidic NK1 selective
e absence of motor dysfunction. Life

he antinociceptive effects of pre- and
MK801, an NMDA antagonist, on the
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CHAPTER 2

ANIMAL MODELS OF PAIN

ALAN COWAN
Temple University School of Medicine
Philadelphia, Pennsylvania

What type of test is most commonly used in preclinical pharmacological research? Procedures involving the measurement of nociception and its suppression are likely contenders, given the regular appearance of pain-related reports in the scientific literature. Overtly simple tests from the 1940s (e.g., hot plate, tail flick) that established the antinociceptive potencies of early opioids are still in use today. The chemical nature of new analgesics has certainly changed over the years, yet the hot plate test links the assessment of meperidine by Woolfe and Macdonald in 1944 with the evaluation of, say, the delta agonist [D-Pen^2, D-Pen^5]enkephalin by Sora and colleagues in 1997. Another example: the tail flick procedure is the enduring constant, linking definitive assays of morphine and codeine in white mice (Wirth, 1952) with current comparisons of morphine and methadone in CXBK counterparts that are naturally insensitive to systemic morphine (Chang et al., 1998). Indeed, both methods (along with the phenylquinone writhing assay) continue to provide initial antinociceptive data on the assorted compounds submitted to the College on Problems of Drug Dependence for evaluation as potentially "nonaddicting" analgesics (e.g., Aceto et al., 1998).

It might be expected that after five decades of experience with these well-established tests, few practical problems would remain. This is not the case. Debate over what aspect of animal behavior represents the most appropriate nocifensive response and how the response should be quantified bedevils the whole field of analgesiometry. For example, rats can respond in a number of ways to the heat stimulus provided by the hot plate. Licking of either the forepaws or hindpaws, lifting paws off the hot surface, jumping, and squeaking represent possible end points. Some researchers record latency to the first appearance of *any* of these signs (e.g., Plone et al., 1996). Others question the equivalence of the behaviors and recommend the more exacting end points of latency to hindpaw lick or vertical jump (Hammond, 1989; Carter, 1991).

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Another ongoing debate is concerned with the relevance of drug-induced changes in tail skin temperature in interpreting data from tail flick tests (Hole and Tjølsen, 1993; Lichtman et al., 1993; Roane et al., 1998). Even an issue as basic as selecting the arbitrary cutoff time in thermal assays, first suggested by Harris and Pierson in 1964 and adopted by countless researchers since then, has been recently questioned by Carmody (1995). Thus the common practice of calculating the "maximum possible effect" (MPE) according to the following formula for each dose of test agent varies with the particular cutoff time chosen and is therefore deemed (correctly) to be "a deeply flawed concept":

$$\%\text{MPE} = \frac{(\text{Test latency} - \text{Control latency})}{(\text{Cutoff latency} - \text{Control latency})} \times 100$$

NATURE OF THE NOCICEPTIVE STIMULUS

The intensity and modality of the noxious stimulus are two experimental variables that have been investigated extensively by analgesic researchers. It is noteworthy that Woolfe and Macdonald (1944), in their pioneering study with the hot plate, examined the activity of test compounds in mice with the zinc plate set at four different temperatures (55°–70°C). More recently, findings from the following groups are of particular importance in the search for alternative analgesics to morphine, for example, agonists at kappa opioid receptors (Rajagopalan et al., 1992).

Shaw and colleagues (Delaney et al., 1986; Shaw et al., 1988) documented the subcutaneous potencies of standard opioids in several pain models and concluded that the mouse abdominal constriction test (0.4% acetic acid) was most sensitive to the opioids, and the mouse (55°C) hot plate test was least sensitive; rat tail flick and rat paw pressure tests were comparable and of intermediate rank. One conclusion to emerge from this work is well-recognized and can be restated here: the stimulus associated with the traditional mouse hot plate test is too high for satisfactory evaluation of many moderate-efficacy kappa agonists (e.g., enadoline, Hunter et al., 1990).

Parsons and Headley (1989) conducted electrophysiological studies with opioids in spinalized, anesthetized rats and demonstrated the importance of intensity of the noxious stimulus, rather than the particular modality used. These workers monitored mu- and kappa-induced suppression of firing by spinal motoneurons elicited by *matched* intensity thermal or pressure stimuli. Under these special circumstances, intravenous kappa agonists had similar effects to fentanyl (a standard mu agonist) against heat and pressure. The key concept of matching the intensity of noxious stimuli in analgesic testing was developed by Millan (1989), who examined mu and kappa agonists in rat tail flick and tail pressure procedures. Subcutaneously administered kappa agonists were equipotent with morphine and fentanyl against moderate, matched-intensity heat and pressure stimuli. Stimuli termed "moderate" were matched in the

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sense that a particular rat responded to both heat and pressure within 3.5–4.4 sec. Interestingly, the kappa agonists (U-50488 and tifluadom) differed from morphine and fentanyl by failing to suppress rat squeaking when electrical stimulation (a stimulus of high intensity) was applied to the animal's tail. Note, however, that subcutaneous administration of U-50488 and tifluadom *are* antinociceptive against this particular noxious stimulus under different experimental conditions, for example, when the rat's tooth pulp is stimulated electrically and the jaw opening response is monitored (Steinfels and Cook, 1986).

It is now clearly established that lowering the intensity of the noxious stimulus can uncover antinociceptive activity for a variety of compounds in, for example, hot plate (Ankier, 1974; O'Callaghan and Holtzman, 1975; Zimet et al., 1986) and tail flick/dip tests (Gray et al., 1970; Granat and Saelens, 1973; Luttinger, 1985; but see Seguin et al., 1995). The experimental strategy of lowering the temperature of the nociceptive stimulus has been taken to the extreme by Pizziketti et al. (1985), who described a cold water adaptation of the rat tail dip test. A 1:1 solution of ethylene glycol and water cooled to −10°C (in a cold water circulating bath) served as the noxious stimulus. [D-Pen^2, D-Pen^5]enkephalin (DPDPE), when given intracerebroventricularly to rats, was active in this procedure but gave an unimpressive dose–response curve in the (50°C) hot water version of the test (Adams et al., 1993). This differential finding may represent a notable characteristic of delta agonists and should be studied further with additional compounds of this class. This comment also applies to dynorphin A, an endogenous kappa agonist that displays the same contrasting effect as DPDPE in the two tests (Tiseo et al., 1988).

The above introduction provides a general background to the field of nociception and antinociceptive agents. In the remainder of the chapter, recent information is summarized on those animal tests that are currently used to evaluate new analgesics.

ACUTE PAIN

Tests involving acute nociceptive noxious stimulation have been reviewed extensively in the preclinical pharmacological literature. The four key references on acute tests, listed in Table 2.1, can be strongly recommended to both seasoned investigators and newcomers to the field of algesiometry. Thus Taber (1974) presents the strengths and limitations of standard methods involving chemical, thermal, mechanical, and electrical stimuli. Such fundamental aspects of analgesic testing as stimulus intensity (discussed above), route of administration, and choice of data analysis are discussed with reference to the drugs of the time, including morphine, pentazocine, and aspirin. These issues are developed at both practical and scholarly levels by Franklin and Abbott (1989), Hammond (1989), and Dubner (1994), and it is clear that, however "old fashioned" the acute tests may seem nowadays, their appropriate use has been

TABLE 2.1 Preclinical Pain Tests Used in Screening for New Analgesics

Pain Model	Test	Key References
Acute	Hot plate, tail flick, paw pressure, tooth pulp, writhing	Taber (1974), Franklin and Abbott (1989), Hammond (1989), Dubner (1994)
Persistent	Formalin	Tjølsen et al. (1992), Porro and Cavazzuti (1993), Aloisi and Carli (1996)
Chronic	Adjuvant-induced arthritis	Colpaert (1987), Besson and Guilbaud (1988)
Incisional	Paw incision test	Brennan et al. (1996)
Visceral	Colorectal distension	Ness and Gebhart (1988), Gebhart and Sengupta (1995)
Neuropathic	Chronic constriction injury	Bennett and Xie (1988)
	Partial nerve ligation	Seltzer et al. (1990)
	Spinal nerve ligation	Kim and Chung (1992)
	Streptozotocin diabetes	Courteix et al. (1993)

responsible for the initial selection and subsequent therapeutic exploitation of today's analgesics (Table 2.2). Of course, the pessimistic view would be that these procedures (although still helpful in the study of pain processes per se) have served their purpose in providing clinically useful drugs, but new approaches are now necessary to discover novel analgesics with different mechanisms of action. As indicated below, the unveiling of new animal models of, for example, neuropathic pain (not mentioned in reviews of the 1980s), along with the discovery of compounds that are active in such models, have maintained the brisk pace of research into analgesics throughout the 1990s.

Methodological Updates

The rhesus monkey warm water (50–55°C) tail withdrawal test was originally introduced by Dykstra and Woods (1986) to allow direct comparison of analgesic-induced effects on nociception, respiration, urine flow, self-administration, drug discrimination, and physical dependence in the same species. Good antinociceptive efficacy has been demonstrated recently for mu (alfentanil) and kappa (enadoline) agonists when the water was maintained at 55°C (France et al., 1994); dynorphin A-(1-13) (Butelman et al., 1995a), butorphanol (Butelman et al., 1995b), and SNC 80, the nonpeptidic delta agonist (Negus et al., 1998), were much less efficacious.

creening for New Analgesics

Key References
Taber (1974), Franklin and Abbott (1989), Hammond (1989), Dubner (1994)
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TABLE 2.2 Antinociceptive Tests Reported in Primary Papers on (Representative) Marketed Analgesics

Analgesic	Class	Test/Activity	Reference
Butorphanol	Opioid agonist–antagonist	Hot plate (+) Skin twitch (−) Tail flick (±) Writhing (+)	Pircio et al. (1976)
Buprenorphine	Mu opioid partial agonist	Tail flick (±) Tail pressure (+) Writhing (+)	Cowan et al. (1977)
Nalbuphine	Opioid agonist–antagonist	Hot plate (−) Writhing (+)	Errick and Heel (1983) Schmidt et al. (1985)
Bromfenac[a]	NSAID	Antibradykinin (+) Tail clip (−) Writhing (+)	Sancilio et al. (1987)
Tramadol	Atypical opioid	Hot plate (+) Tail flick (+) Writhing (+)	Raffa et al. (1992)
Meloxicam	COX-2 preferring NSAID	Hot plate (−) Inflamed paw pressure (+) Tail clip (−)	Engelhardt et al. (1995)

[a]Withdrawn in June 1998 because of drug-induced liver toxicity.

Dilute solutions of acetic acid and phenylquinone are injected intraperitoneally in mice to precipitate writhing/stretching in the popular abdominal constriction test. Observing the mice can be tedious and time consuming. Adachi (1994) has described a mechanoelectro transducer that automatically and objectively counts each writhe after the acetic acid. There was a good correlation between results from human observation and from the detecting unit when standard agents were assayed.

Dilute solutions of acetic acid have also been used by Stevens and colleagues to demonstrate the antinociceptive potencies of opioids, given systemically, spinally, or supraspinally to northern grass frogs (Stevens et al., 1994; Stevens, 1996; Stevens and Rothe, 1997). The test involves placing a single drop from one of 10 dilutions of acetic acid on the frog's hindlimb and observing the frog for 5 secs for a wiping response. In the absence of a response (e.g., after morphine administration), the acid is flushed away with water and the next dilution is tested, and so on until a wiping response occurs. On the basis of studies such as these, Stevens is advancing a case (on both ethical and economic grounds) for replacing rodents with frogs when new opioid agents are to be screened.

The intraperitoneal injection of endothelin-1 (ET-1) into mice causes the animals to writhe as if given acetic acid or phenylquinone. Raffa and colleagues (1996) believe, however, that the underlying mechanisms differ. Their evidence is based, partly, on differential antinociceptive ED_{50} values for 36 standard compounds tested against equieffective doses of the three noxious agents. Of particular note was the good activity of diazepam and several other benzodiazepines against (specifically) ET-1-elicited writhing. The procedure holds promise as a model for ischemic pain of visceral organs and for the discovery of compounds that otherwise are inactive in conventional writhing assays.

PERSISTENT PAIN

Formalin Test

Dubuisson and Dennis (1977) are usually given credit for bringing the rat formalin test to the attention of pain researchers. They emphasized the continuous (rather than transient) nature of the noxious stimulus and the opportunity for the animals to behave spontaneously. The injection of a dilute solution of formalin into the hindpaw of a rat elicits at least two nocifensive behaviors: flinching/shaking of the paw and/or hindquarters and licking/biting of the injected paw (Wheeler-Aceto et al., 1990). The behavioral response to formalin is biphasic. An acute/early phase lasting about 10 min is caused by activation of peripheral nociceptors. This is followed, after a short quiescent period, by a tonic/late phase persisting from 20 to 90 min after injection and linked to ongoing activity in primary afferents and increased sensitivity of dorsal horn neurons. The tonic/late phase is initiated, at least in part, by activation of *N*-methyl-D-aspartate (NMDA) receptors in the spinal cord (Dickenson and Sullivan, 1987; Haley et al., 1990).

Formalin-induced hyperalgesia represents a behavioral model of tonic chemogenic pain. The procedure has involved the use of mice (e.g., Murray et al., 1988; Shibata et al., 1989; Mogil et al., 1998), rats (Wheeler-Aceto and Cowan, 1991; Chaplan et al., 1997; Hammond et al., 1998), gerbils (Smith et al., 1994; Rupniak et al., 1996; Chapter 7 of this volume), cats (Dubuisson and Dennis, 1977), and rhesus monkeys (Alreja et al., 1984), and practical issues relevant to the model have been reviewed succinctly by Tjølsen and colleagues (1992). A simple modification, involving the injection of formalin into the vibrissal pad of rats and monitoring the duration of lip rubbing, has facilitated the study of orofacial pain and its suppression by drugs (Clavelou et al., 1989, 1995; Eisenberg et al., 1996).

The formalin test has grown enormously in popularity over the last decade and it is arguably the most commonly used model of postoperative pain in current analgesic research. An indication of this acceptance is displayed in Figure 2.1, where the papers that have appeared in the journal *Pain* and in

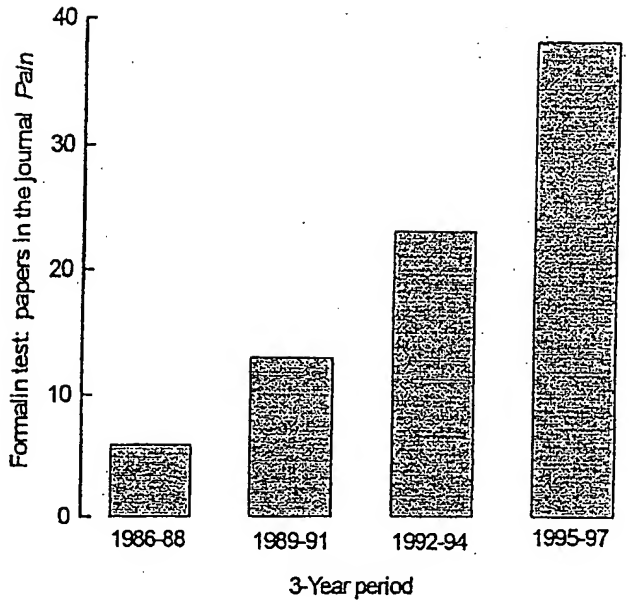

Figure 2.1 The number of papers describing the formalin test that were published in the journal *Pain* in 3-year periods between 1986 and 1997.

which the formalin test is featured are shown in 3-year histograms since 1986. The steady rise in use across time is apparent.

Methodological Update

The numerous variables associated with evaluation of antinociceptive activity in traditional (acute) procedures (Hammond, 1989) are also of concern in the formalin model. Thus such well-recognized factors as strain, age, and stress level of test animal, site/route of injection, and choice of data analysis can all influence the shape and position of a compound's dose–response curve against the formalin noxious stimulus. A perennial criticism of phasic pain studies can also be directed at accumulating data from the formalin test: how meaningful is an antinociceptive-50 value that depends entirely on a top dose of "analgesic" inducing behavioral depression and/or motor dysfunction in the animals? Or again, is the noxious stimulus (formalin) standardized such that findings may be compared across laboratories? This latter issue is considered in the following section.

Formalin Concentration and Nocifensive Behaviors. Commercially available formaldehyde solution (Merck and Co.) contains 36–38% formaldehyde along with 10–15% methanol (which acts as a stabilizer). A 1% formalin solution is made with 0.1 ml of formaldehyde in 9.9 ml of water (or,

more usually, saline) so that the formaldehyde concentration is roughly 0.37% (Teng and Abbott, 1998).

Use of formalin concentrations between 1 and 5% has been recommended when studying drug-induced antagonism of tonic/late phase licking/biting in mice (Hunskaar et al., 1985; Murray et al., 1988; Rosland et al., 1990). On the basis of several recent reports, 20 μl of 2.5 or 5% formalin injected subcutaneously into either the dorsal or plantar surface of a mouse hindpaw is becoming the standard noxious stimulus in this test (e.g., Millan and Seguin, 1994; Noble et al., 1995; Wettstein and Grouhel, 1996; Bittencourt and Takahashi, 1997; Rupniak et al., 1997).

With rats, the choice of formalin concentration is no trivial matter and can influence the outcome of an assay, particularly if non-morphinelike agents are being screened (Poon and Sawynok, 1995). For example, in the more commonly monitored late phase, nociceptive responses to 50 μl of 5% formalin, but not to 50 μl of 1% formalin, were suppressed in a dose-related manner by intraperitoneal administration of the anti-inflammatory drugs ibuprofen (40–250 mg/kg) and dexamethasone (1–6.25 mg/kg) (Yashpal and Coderre, 1998). The stimulus intensity–drug response relationship can also work in the opposite direction; thus the potency of intraperitoneal caffeine against late phase flinching is increased by a factor of 8 when 20 μl of 5% formalin is replaced with 20 μl of 2% formalin (Sawynok and Reid, 1996). It is therefore prudent to study new compounds against at least two concentrations of formalin before reaching a conclusion on antihyperalgesic activity in the test. Concentrations of formalin within the 1–5% range would seem to be reasonable choices if either the incidence of late phase flinching (Ossipov et al., 1996; Dirig et al., 1997a) or a weighted-scores measure (Dubuisson and Dennis, 1977; Matthies and Franklin, 1992) is used to quantify nociceptive behavior. This is because both flinching and weighted-scores increase in a linear fashion up to the submaximal concentration of 5% formalin (Coderre et al., 1993; Wheeler-Aceto and Cowan, 1993; Jett and Michelson, 1996; but see Abbott et al., 1995 and Watson et al., 1997).

Two automated systems have been reported recently to provide an objective measure of formalin-induced nociceptive behavior in rats. Jett and Michelson (1996) described a computer-driven, dynamic-force detector that specifically quantifies nociceptive "agitation" behaviors (flinching and licking/biting) of rats placed in small polycarbonate observation tubes (Figure 2.2). A good correlation was found between the agitation response and two manual assessments: incidence of flinching and the weighted-scores measure of Coderre et al. (1993). Jourdan and colleagues (1997) described an automated system (Videotrack) based on computerized image processing that also measured pain-related behaviors (licking, biting, grooming), as well as motor activity, of rats allowed to explore a large plastic chamber. The authors validated their procedure against the weighted-scores measure of Dubuisson and Dennis (1977). It remains to be seen to what extent these automated devices will be used in pain research and drug discovery.

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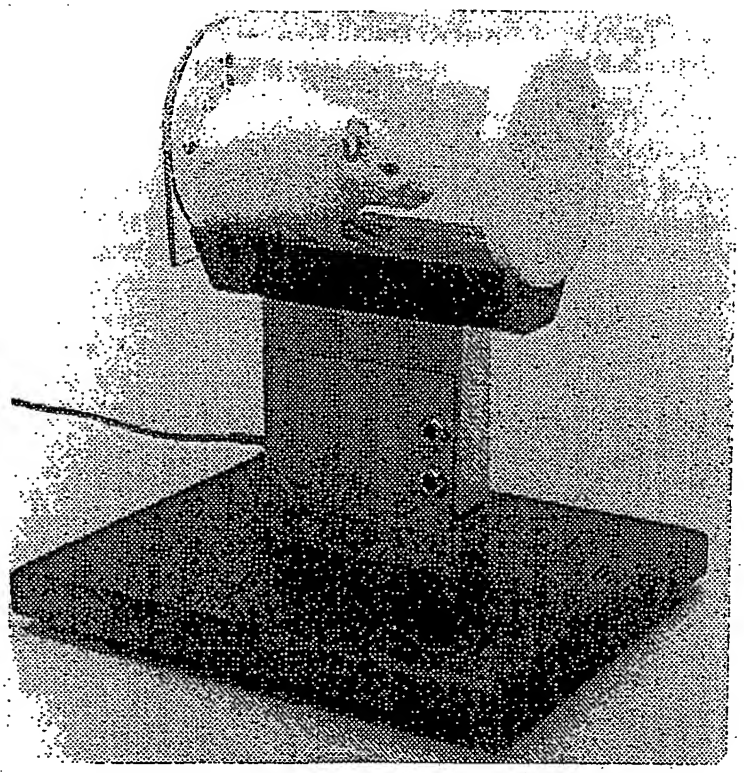

Figure 2.2 A rat (125–170 g) in an observation tube (16 cm long; 8 cm wide) positioned in the cradle of a load cell of the automated behavioral measuring system. Reproduced from Jett and Michelson (1996), with permission of the publisher. A commercial version that monitors up to 24 animals at a time is available from AccuScan Instruments (Columbus, Ohio).

Pharmacological Update

Antihyperalgesic activity has been claimed for a formidable array of compounds in the mouse/rat formalin test, particularly in the late phase. For example, in addition to positive findings with mu, kappa, and delta opioids (Pelissier et al., 1990; Murray and Cowan, 1991; Hammond et al., 1998) and several currently marketed analgesics (Table 2.3), recent results indicate activity for bradykinin antagonists (Corrêa and Calixto, 1993; Corrêa et al., 1996; Sufka and Roach, 1996), capsaicin analogues (Dray and Dickenson, 1991; Hua et al., 1997), adenosine analogues (Poon and Sawynok, 1995; Reeve and Dickenson, 1995), γ-aminobutyric acid agonists (Dirig and Yaksh, 1995; Kaneko and Hammond, 1997), nonsteroidal anti-inflammatory agents (NSAIDs) (Carrive and Meyer-Carrive, 1997; Euchenhofer et al., 1998; but see Dirig et al., 1997a), antidepressants (Acton et al., 1992; Jett et al., 1997) and antiepileptics (Field et al., 1997b; Shimoyama et al., 1997; Carlton and Zhou, 1998; and quoted in Nakamura-Craig and Follenfant, 1995).

TABLE 2.3 Potencies of Analgesics against Severe (Cancer) Pain in Humans and against Formalin-Induced Flinching in Rats

Analgesic	Equianalgesic Dose (mg, i.m.)[a]	A_{50} (mg/kg, s.c.)[b]
Buprenorphine	0.4	0.03 (0.02–0.04)
Butorphanol	2	0.14 (0.02–0.39)
Morphine	10	0.58 (0.47–0.71)
Nalbuphine	10	Curvilinear DRC; maximum antihyperalgesic effect = 57%
Pentazocine	60	0.92 (0.66–1.2)
Ketorolac	10–30[c]	Curvilinear DRC; maximum antihyperalgesic effect = 47%
Tramadol	[d]	2.1 (1.3–3.0) p.o.
Codeine	200 p.o.	25.8 (16.5–41.6) p.o.

[a]Foley (1985).
[b]Cowan et al. (1990) and Wheeler-Aceto and Cowan (unpublished results). A_{50} values and 95% confidence limits were determined by linear regression analysis from the percentage antagonism of formalin-induced (late phase) flinching.
[c]Staquet (1989); Eisenberg et al. (1994).
[d]Under study (Budd, 1995).
DRC = dose–response curve.

Two classes of pharmacological agent have been studied extensively in the formalin test; antagonists at neurokinin (NK) receptors (Seguin et al., 1995; Iyengar et al., 1997; Sakurada et al., 1997) and NMDA receptor antagonists (Kristensen et al., 1994; Elliott et al., 1995). It has been quite difficult to demonstrate *specific* antinociceptive activity for NK_1 receptor antagonists against the formalin noxious stimulus (Rupniak et al., 1995). This was a portent, perhaps, of the disappointing results obtained with these compounds in clinical trials involving migraine and dental pain (Chapter 7 of this volume). Several competitive and noncompetitive NMDA receptor antagonists are unimpressive in acute nociceptive procedures yet are active in the late phase of the formalin test (Chaplan et al., 1997; Chapter 8 of this volume). Unfortunately, the association of motor dysfunction in the animals with some of the higher intrathecal doses under test has been a confounding factor when attempting to establish dose–response relationships (Coderre and Van Empel, 1994). Nonetheless, despite concern over a narrow therapeutic window, two well-known NMDA receptor antagonists—dextromethorphan (the antitussive) and memantine (the antiparkinsonian agent)—have progressed through clinical trials. Dextromethorphan will be used primarily for treating cancer pain (when given in combination with morphine) and possibly arthritic pain (when given in combination with an NSAID) (Price et al., 1996). The indication for memantine is neuropathic pain. For present purposes, note the key role that the formalin test is playing in the primary evaluation of tomorrow's analgesics from the compound activity profiles listed in Table 2.4.

evere (Cancer) Pain in Humans
ts

A_{50} (mg/kg, s.c.)[b]
0.03 (0.02–0.04)
0.14 (0.02–0.39)
0.58 (0.47–0.71)
Curvilinear DRC; maximum antihyperalgesic effect = 57%
0.92 (0.66–1.2)
Curvilinear DRC; maximum antihyperalgesic effect = 47%
2.1 (1.3–3.0) p.o.
25.8 (16.5–41.6) p.o.

unpublished results). A_{50} values and 95% a s from the percentage antagonism

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INCISIONAL PAIN

Paw Incision Test

A new model of postoperative pain involving rats has been introduced recently by Brennan et al. (1996) and validated pharmacologically by Zahn et al. (1997). The rat is anesthetized, a 1 cm longitudinal surgical incision is made through skin, fascia, and muscle in the plantar area of the animal's hindpaw and the wound is then closed. Tactile allodynia (i.e., pain due to a stimulus that does not normally provoke pain), revealed through the paw withdrawal response to calibrated von Frey filaments as well as nonpunctate and pinprick responses, ensues and this hypersensitive state lasts for several days after the incision. It is perhaps surprising that an animal model endowed with such obvious face validity (though not in itself an absolute criterion) has taken so long to appear in the analgesic literature. At any rate, morphine, given either subcutaneously or intrathecally to the rats, was active in the model (Zahn et al., 1997), as were intrathecal 6,7-dinitroquinoxaline-2,3-dione (DNQX) and 1,2,3,4-tetrahydro-6-nitro-2,3-dioxo-benzo[f]quinoxaline-7-sulfonamide disodium (NBQX), two standard non-NMDA excitatory amino acid receptor antagonists (Zahn et al., 1998). Importantly, from a predictive point of view, neither a competitive (dizocilpine) nor a noncompetitive (2-amino-5-phosphonovaleric acid) NMDA receptor antagonist was active against incisional pain at behaviorally acceptable intrathecal doses (Zahn and Brennan, 1998). The authors conclude that NMDA receptor antagonists, per se, are unlikely to be useful for pain relief after surgery.

Additional compounds are currently being evaluated in the procedure. Thus gabapentin (Field et al., 1997a), the antiepileptic agent known to be active against formalin (Singh et al., 1996) and in models of neuropathic pain (see below) (Xiao and Bennett, 1996; Hwang and Yaksh, 1997), as well as ziconotide (Table 2.4) (Wang et al., 1998) gave positive results against incisional pain. PD 154075, the selective NK_1 receptor antagonist (Gonzalez et al., 1998), was given subcutaneously to rats either pre- or postoperatively. This compound was active in the test only when given *before* the surgery.

VISCERAL PAIN

Colorectal Distension Test

Instilling irritants such as resiniferatoxin (Craft et al., 1995) and turpentine (McMahon and Abel, 1987) into the urinary bladder and distending the hollow viscera (Borgbjerg et al., 1996), are two increasingly common ways of evoking visceral nociception in animals. The testing of analgesics in unanesthetized rats using minimally invasive distension of the descending colon and rectum has become a well-recognized standard approach (Ness and Gebhart, 1988; Burton and Gebhart, 1998). Colorectal distension is effected by means of an intra-

TABLE 2.4 Representative Analgesics in Clinical Development and Pain Models in Which They Show Activity

Compound	Pharmacological Class	Formalin Test[a] (1)	CCI[b] Model (Rat) (2)	SNL[c] Model (Rat) (3)	Clinical Status
ABT-594[d] (Abbott)	Nicotinic acetylcholine receptor agonist	Flinching, licking, biting (i.p.) (4)	Tactile allodynia (i.p.) (4)	n/a	Phase I
Asimadoline (E. Merck)	Peripherally selective kappa agonist	Licking (s.c., p.o.) (5)	n/a	n/a	Rheumatic pain/ osteoarthritis—Phase II
Clonidine (Roxane)	α-Adrenergic agonist	Flinching (i.th.) (6)	Heat hyperalgesia (i.th.) (7)	Tactile allodynia (i.th.) (8)	Marketed as Duraclon™ for epidural use in combination with opiates against cancer pain
Dextromethorphan (Algos)	Noncompetitive NMDA antagonist	Flinching, licking (s.c.) (9)	Heat hyperalgesia (with i.th. dextrorphan, the metabolite) (10)	Tactile allodynia (i.th.) (11)	In combination with morphine (as MorphiDex™) for cancer pain—new drug application filed